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(57) Abstract

The present invention describes a method for the selection and temporal expression of heterologous genes. The invention consists of methods to obtain selective and temporal expression of heterologous genes in target tissues. Promoters inducible by photodynamic therapy or heat are used to express genes of interest under conditions of heating or Photodynamic Therapy–(PDT–) induced oxidative stress. Selective and temporal expression of heterologous genes (such as cytokines, toxins, tumor suppressor genes, antisense molecules and anti–angiogenic factors) are of significant therapeutic benefit in the treatment of tumors, vascular proliferation and tissue hypertrophy. Gene therapy targeted by laser induced heating, other heating sources (such as microwave, ultrasound or radiofrequency induced currents), or PDT enhances treatment effectiveness by inducing expression of therapeutic genes in a controlled and localized manner.

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PHOTODYNAMIC THERAPY GENERATED OXIDATIVE STRESS FOR TEMPORAL AND SELECTIVE EXPRESSION OF HETEROLOGOUS GENES

BACKGROUND OF THE INVENTION

This invention was produced in part using funds obtained through a grant from the National Institutes of Health. Consequently, the federal government has certain rights in this invention.

Field of the Invention

The present invention relates generally to the clinical treatment of solid tumors. More specifically, the present invention relates to inducing expression of therapeutic genes in a controlled and localized manner.

Description of the Related Art

Photodynamic therapy (PDT) is a clinical treatment of solid malignancies (Fisher, A.M.R., et al., *Laser Surgery Medicine* 17:2-31 (1995); Marcus, S.L. and Dugan, M.H., *Laser Surgery Medicine*, 12: 318-24 (1992); and Henderson, B.W. and Dougherty, T.J., *Photochem. Photobiol.*, 55:931-48 (1992)). Properties of photosensitizer localization in tumor tissue and photochemical generation of reactive oxygen species are combined with precise

delivery of laser generated light to produce a procedure offering effective local tumoricidal activity (Henderson, B.W., *Photodermatology* 6:200-11 (1989); and Wilson, B.C., and Jeeves, W.P., PHOTOMEDICINE (Ed. by Ben Hur, E., and I. Rosenthal) 2:127-67 (1987)). A derivative of hematoporphyrin termed Photofrin is the photosensitizer used in the majority of clinical trials. Photofrin-mediated PDT has recently received FDA approval for treatment of esophageal carcinoma and this compound has also received regulatory approval in Canada, the Netherlands and Japan. PDT shows considerable promise for treating tumors of the bronchus, bladder, skin, head/ neck and cervix as well as for non-malignant disorders such as psoriasis and age-related macular degeneration.

Growing interest exists also in the development and clinical evaluation of new photosensitizers exhibiting improved pharmacological, photochemical and photophysical properties (Gomer, C.J., *Yearly Review: Photochem. Photobiol.*, 54:1093-1107 (1991)). Second generation photosensitizers undergoing clinical trials include tin etiopurpurin (SnET2), mono-l-aspartyl chlorin e6 (NPe6), benzoporphyrin derivative (BPD), meso-tetra-(hydroxyphenyl) chlorin (mTHPC) and 5-amino levulinic acid (ALA). These compounds exhibit a number of properties thought to be comparable or superior to Photofrin including chemical purity, increased photon absorption at longer wavelengths, improved tumor tissue retention, rapid clearance from normal tissues, high quantum yields of reactive oxygen species and minimal dark toxicity (Dougherty, T.J., *Photochem. Photobiol.* 58:895-900 (1993); Moan, J. and Berg, K., *Photochem. Photobiol.* 55:931-48 (1992) and Kessel, *Photochem. Photobiol.*, 50:169-74 (1989)). ALA is a metabolic precursor of endogenous protoporphyrin IX.

Photochemical generation of reactive oxygen species during PDT can produce damage to lipids, proteins and nucleic acids (Moan, J., *Photochem. Photobiol.* 43:681-90 (1986)). Indeed, injury to cellular membranes, organelles, enzymes and DNA have been documented in numerous normal and malignant cells (Prinze, C., et al., *Biochim. Biophys. Acta.*, 1038:152-57 (1990); and Hilf, R., et al., *Cancer Res.*, 44:1483-88 (1984)). Oxidative damage to any

of these sites could be sufficient to cause cell death. However, the actual mechanism(s) and target(s) of PDT-induced lethality remain to be elucidated.

PDT induces expression of early response genes (c-fos, c-jun, c-myc, egr-1) as well as stress protein genes belonging to the heat shock proteins (HSP), glucose regulated proteins (GRP) and heme oxygenase families (Luna, M.C., et al, *Cancer Res.*, 54:1374-80 (1994); Gomer, C.J., et al., *Photochem. Photobiol.*, 53:275-279 (1991); Gomer, C.J., et al., *Cancer Res.*, 51:6574-79 (1991); and Gomer, C.J., et al., *Cancer Res.* (In press) (1997)). Induction of the stress genes is at the level of transcription but information on specific targets and/or signaling pathways responsible for inducting stress responsive gene expression is unknown. Apoptosis is also induced by PDT and appears to involve a signal transduction pathway originating at the cell membrane (Agarwal, M.L., et al, *Cancer Res.*, 51:5993-96, (1991); He, X.Y., et al., *Photochem. Photobiol.* 59:468-73 (1994) and Zaidi, S.I.A., et al., *Photochem. Photobiol.* 58:771-76 (1993)). Characteristic DNA fragmentation, chromatin condensation, and activation of a constitutive endonuclease is observed in photosensitized cells. Apoptosis has also been identified *in vivo* as an early event in PDT-treated tumors.

Cells have different stress responses. Each such response is triggered by one or more kinds of environmental insult or stress, the main consequence of its induction being increased stress tolerance levels. The heat shock response is one of the best-characterized of these stress responses. In 1962, it was reported that exposure to high but nonlethal temperatures (heat shock) and to certain chemicals causes the appearance of new puffs on the salivary gland *Drosophila busckii*. These puffs, clearly visible in the light microscope, represent high transcriptional activity on heat shock-inducible genes, and RNA synthesis that generates the messenger RNAs for the heat shock proteins. More recently, a wide range of organisms, from bacteria to higher vertebrates, has been shown to display similar dramatic changes in gene expression with heat shock.

The response to heat shock entails both strong induction of genes for heat shock proteins (HSPs) and repression of most genes that were being expressed previous to the induction. There are marked changes both to patterns of gene transcription and to the way in which mRNAs are selected for translation by the protein-synthesizing machinery of the cell.

The transcriptional changes with heat shock are due to the presence of a heat shock element in heat shock gene promoters. This element is a DNA sequence needed for specific induction of transcription in response to heat shock. In the heat-inducible promoters of *E. coli*, the concensus sequence for the heat shock element is CTGCCACCC at nucleotide positions -44 to -36 relative to the transcription initiation site. In the heat-inducible promoters of eukaryotes, it is contiguous repeats of the 5-bp sequence NGAAN, arranged in alternative orientation positioned upstream of the TATA box element.

For experimental purposes, the heat shock response is usually induced by a temperature upshift. The optimal temperature for induction is species dependent, but it is usually a degree or two above the maximum that permits growth. Also, the response is generally transient.

There are other inducers of heat shock protein synthesis besides thermal stress. These include several potentially cytotoxic chemicals and physiologic states which may cause generation of highly reactive free radicals. These inducers likely share with high temperatures the ability to cause intracellular accumulation of aberrant or partially denatured protein.

Glucose Regulated Proteins, or GRPs, with molecular weights of 78,000 and 94,000, share sequence homology with heat shock proteins. The GRP family of proteins is coordinately induced by glucose starvation, anoxia, alterations in intracellular calcium, exposure to inhibitors or glycosylation as well as by PDT-mediated oxidative stress (Gomer, C.J., et al., *Cancer Res.* 51:6574-79 (1991); and Li, L-J., et al., *J. Cell Physiol.* 153:575-82 (1992)). The 78,000 GRP is identical in sequence to the immunoglobulin heavy chain binding protein and both GRP78 and GRP94 are localized in the endoplasmic reticulum (ER). GRP78 binds transiently to nascent,

secretory and transmembrane proteins and binds permanently to abnormally folded or processed proteins in the ER. GRP78 is thought to have a protective function during and after cellular stress when protein processing in the ER is perturbed. Lee et al. have shown that transcriptional activation of grp78 by tumor hypoxia can be exploited for targeted gene therapy. A truncated grp78 promoter with most of the basal elements removed was shown effectively to drive high level expression of a reporter gene in hypoxic mouse tumors (Gazit, G., et al., *Cancer Res.* 55:1660-63 (1995)).

The present invention demonstrates that PDT-mediated oxidative stress is a strong transcriptional transducer of stress proteins, specifically those belonging to the heat shock proteins. Thus, the present invention is drawn to methods of targeted gene therapy using recombinant constructs with HSP- or GRP-inducible promoters to drive high-level, local expression of cytotoxins or immunomodulators to enhance PDT tumoricidal action.

Thus, the prior art is deficient in methods of producing high level local expression of cytotoxins or immunomodulators. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a novel method for enhancing locally the tumoricidal (or anti-angiogenic) properties of PDT (or heat) by using these therapeutic modalities to act as a molecular switch for the spatial and temporal expression of genes to enhance or act synergistically with the direct effects of PDT. The present invention is superior to current strategies which attempt to deliver localized cytotoxic gene therapy using constitutively-acting promoters, i.e. viral delivery systems using tissue-specific receptors or tissue-specific enhancers that limit gene transcription to a select group of cells. The temporal regulation of gene expression is not possible using constitutively-acting promoters due to the high basal expression of cytotoxic gene productions in normal cells and tissue. The present invention (combining inducible gene therapy and PDT or

heat) enhances local tumor control without increasing normal tissue toxicity (increasing the therapeutic gain). Transcriptional activation of a PDT or heat inducible promoter is controlled within a given tissue volume and for a specific time period. This procedure, therefore, exploits both the direct cytotoxicity induced by PDT or heat and the targeting potential of PDT or heat to induce spatial and temporal regulation of cytotoxic gene transcription.

PDT- or heat-targeted gene therapy uses a construct containing a PDT- or heat-inducible promoter upstream of a cDNA encoding a heterologous gene which is activated transcriptionally within the PDT or heat treatment field to enhance local cytotoxicity. Weichselbaum et al. has reported on a similar procedure using ionizing radiation (Weichselbaum, R.R., et al., *Cancer Res.* 54:4266-69 (1994)).

One object of the present invention is to provide a method of temporal and localized treatment of a target tissue in an individual comprising the steps of: administering an expression vector to said individual, wherein said vector expresses a therapeutic, heterologous gene under control of a promoter inducible by photodynamic therapy or heat, and exposing said target tissue to said photodynamic therapy or heat. Various embodiments of this particular object of the invention include generating heat by thermal laser, microwaves, ultrasound or radiofrequency waves.

In yet another object of the present invention, there is provided a method of temporal and localized treatment of a target tissue in an individual comprising the steps of: administering a photosensitizer for photodynamic therapy to said individual; administering an expression vector to said individual, wherein said vector expresses a therapeutic, heterologous gene under control of a promoter inducible by photodynamic therapy; allowing said photosensitizer and said expression vector to be taken up by said target tissue; and exposing said target tissue to light, wherein said light combined with said photosensitizer will generate reactive oxygen species to induce said promoter inducible by photodynamic therapy, causing said therapeutic, heterologous gene to be expressed. A particular embodiment of this object of the present invention includes a photosensitizer is

selected from the group of photofrin, tin etiopurpurin, mono-l-aspartyl chlorin e6, benzoporphyrin derivative, meso-tetra-(hydroxyphenyl)chlorin and 5-amino levulinic acid.

Various embodiments of both objects of the method of the present invention include administering the vector systemically or locally. Preferred embodiments of the vector of the present invention include cases wherein said vector is a retroviral vector, an adeno-associated viral vector, or a liposomal DNA vector.

Particular preferred embodiments of both objects of the present invention are where said promoter is a heat shock protein (hsp) promoter or a glucose regulated protein promoter. Further, preferred embodiments include wherein said heterologous gene is an immunomodulatory gene, particularly a cytokine. In addition, embodiments include where said heterologous gene is a tumor suppressor gene, an anti-sense DNA, or an anti-angiogenic gene.

In either object of the present invention, a preferred embodiment includes where said target tissue is a tumor or an area of abnormal tissue growth.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The appended drawings have been included herein so that the above-recited features, advantages and objects of the invention will become clear and can be understood in detail. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and should not be considered to limit the scope of the invention. Figures 1-5 demonstrate the selective and temporal expression of the beta-galactosidase (β-gal) reporter gene in RIF HB-3 cells exposed to either heat or PDT:

Figure 1: Beta-galactosidase activity (measured by using ONPG (o-nitrophenyl-pyranogalactose) as a substrate) in transfected RIF HB-3 cells treated with heat (45°C) for 1.5, 10, 20, 30 and 40 minutes. Samples were collected six hours after hyperthermia. Each data point represents the average of at least three separate experiments ± S.E.

Figure 2. Beta-galactosidase activity (measured by using ONPG as a substrate) in RIF HB-3 cells treated with NPe6-mediated PDT at doses ranging from 600 Joules/cm^2 to 5400 Joules/cm^2. Samples were collected six hours after light exposure. Each data point represents the average of at least three separate experiments ± S.E.

Figure 3. Kinetics of Beta-galactosidase expression (measured by using ONPG as a substrate) in RIF HB-3 cells treated at 45°C for 20 minutes. Each data point represents the average of at least three separate experiments ± S.E. Enzyme activity was detected three hours after heat treatment (19 milliunits/mg protein). The peak values of β-gal activity (100 and 125 milliunits/mg protein) were reached at six and twelve hours following heat exposure.

Figure 4. Kinetics of Beta-galactosidase expression (measured by using ONPG as a substrate) in RIF HB-3 cells treated with NPe6-PDT at a light dose of 3000 Joules/cm^2. Each data point represents the average of at least three separate experiments ± S.E. A minimal level of enzyme activity was detected three hours after treatment. Peak activity was reached between six and twelve hours after NPe6 mediated PDT.

Figure 5. Beta-galactosidase activity (measured by using ONPG as a substrate) from RIF HB-3 tumors growing in C3H/HeJ mice and treated with either heat or NPe6-PDT. Heat treatment consisted in 20 minutes exposure at 45 °C. Three doses of light were tested for PDT. Each column represents the average of five distinct measurements ± S.E. The largest response was observed following hyperthermia (8.25 milliunits/mg protein). NPe6-PDT induced β-gal enzyme activity ranged between 1.08 and 3.48 milliunits/mg protein. No measurable levels were recorded for control tumors nor for tumors treated only with light or NPe6. (Comparable results were obtained for PDT treatments using PH and Sn ET2.)

Figures 6 through 12 demonstrate the selective and temporal expression of the chloramphenicol acetyl transferase (CAT) reporter gene in RIF HC-2 cells and RIF RHC-7 cells exposed to either heat or PDT

Figure 6. CAT activity in transfected RIF HC-2 cells treated with heat (45°C) for 1.5, 10, 20, and 40 minutes. Samples were collected 24 hours after hyperthermia. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

Figure 7. CAT activity in transfected RIF RHC-7 cells treated with heat (45°C) for 1.5, 10, 20, and 40 minutes. Samples were collected 24 hours after hyperthermia. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

Figure 8. CAT activity in transfected RIF HC-2 cells treated with NPe6 mediated PDT at doses ranging from 600 Joules/cm^2 to 5400 Joules/cm^2. Samples were collected 24 hours after light exposure. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

Figure 9. Kinetics of CAT activity in transfected RIF HC-2 cells treated at 45°C for 20 minutes. Samples were collected 3-48 hours after heat exposure. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

Figure 10. Kinetics of CAT activity in transfected RIF RHC-7 cells treated at 45°C for 20 minutes. Samples were collected 3-48 hours after heat exposure. Conversion of chloramphenicol to acetylated chloramphenicol was calculated by counting radioactivity from resulting TLC plates.

Figure 11. CAT activity from RIF HC-2 cells and RIF HC-2 tumors growing in C3H/HeJ mice and treated with either water bath heat for cells or laser induced heat for tumors. Heat treatment consisted of 20 minutes exposure at 45 °C. Cell culture samples were collected 24 hours after heating and tumor samples were collected 3, 6 or 24 hours after heating No measurable CAT activity levels were recorded for control tumors.

Figure 12. CAT activity from RIF HC-2 and RIF RHC-7 tumors growing in C3H/HeJ mice and treated with either laser induced heat, NPe6-PDT or PH-PDT. Heat treatment consisted in 20 minutes exposure at 45°C. No measurable levels were recorded for control tumors or for tumors treated only with light, NPe6 or PH.

DETAILED DESCRIPTION OF THE INVENTION

It will be apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "photodynamic therapy" or "PDT" refers to the treatment of solid tumors with visible light (usually generated by non-thermal lasers) following the systemic administration of a tumor localizing photosensitizer (see Fisher, A.M.R., et al., *Laser Surgery Medicine* 17:2-31 (1995); Marcus, S.L. and Dugan, M.H., *Laser Surgery Medicine*, 12: 318-24 (1992); and Henderson, B.W. and Dougherty, T.J., *Photochem. Photobiol.*, 55:931-48 (1992)). The photochemical reaction induced by the photosensitizer and laser light produces reactive oxygen species such as singlet oxygen which in turn induces oxidative damage to subcellular targets (membranes, organelles, enzymes, and DNA). PDT is used clinically to treat various types of solid tumors (esophagus, bronchus, bladder, brain, eye, head/neck, skin, cervical as well as non-malignant diseases such as age related macular degeneration and psoriasis. Various photosensitizers, including Photofrin (PH), tin etiopurpurin (SnET2), mono-l-aspartyl chlorin e6 (NPe6), benzoporphyrin derivative (BPD), meso-tetra-(hydroxyphenyl) chlorin (mTHPC) and 5-amino levulinic acid (ALA) are used in PDT.

As used herein, the term "heat shock gene" refers to a gene which is transcribed at a high level in response to elevated temperature.

As used herein, the term "CAT" or "CAT assay" refers to an assay used to assess *in vivo* effectiveness of eukaryotic promoter

sequences. The CAT gene codes for CAT, which inactivates the antibiotic chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups. Because eukaryotic cells do not synthesize CAT, the gene has been exploited as a reporter gene for analysis of promoters, particularly in mammalian cells. CAT is assayed by a method based on thin-layer chromotography in which [^{14}C]chloramphenicol can be separated from the acetylated and inactive derivatives which are only synthesized in the presence of the CAT enzyme.

As used herein, the term "β-gal" or "β-galactosidase assay" refers to an assay used to assess *in vivo* effectiveness of eukaryotic promoter sequences. β-galactosidase is an enzyme which hydrolyzes β-galactosides such as lactose into component sugars by hydrolysis of terminal nonreducing β-galactose residues. The *E. coli* LacZ gene is used as a reporter gene in studies of promoter action as a translational in-frame fusion between a gene of interest and the LacZ gene puts lacZ expression under the control of the promoter under investigation. The activity of the promoter can then be assayed by measuring the β-galactosidase activity using o-nitrophenyl-pyranogalactose as a substrate.

As used herein, the term "glucose regulated protein" or "GRP" refers to the family of proteins that is induced coordinately by glucose starvation, anoxia, alterations in intracellular calcium, exposure to inhibitors or glycosylation as well as by PDT mediated oxidative stress conditions.

As used herein, the term "tumor suppressor gene" refers to a class of genes believed to be involved in different aspects of normal control of cellular growth and division. The common characteristic of these genes is that it is their inactivation, usually by genetic means, which contributes to tumor development.

As used herein, the term "immunomodulatory gene" refers to a gene the expression of which modulates the course of an immune reaction to a specific stimulus or a variety of stimuli. Examples include interleukin 4, interleukin 10, tumor necrosis factor α, etc.

As used herein, the term "cytokine" refers to a small protein produced by cells of the immune system that can affect and direct the course of an immune response to specific stimuli.

As used herein, the term "anti-angiogenic gene" refers to genes coding for proteins which reduce or terminate the formation of blood vessels.

As used herein the term "antisense DNA" refers to DNA which codes for an antisense RNA. Such antisense RNA has the potential to form an RNA-RNA duplex with the natural 'sense' mRNA transcript of a gene, thereby preventing its translation. Antisense RNA provides a means of inactivating the expression of specific genes and can be applied to both simple and complex eukaryotes.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells And Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984)).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A vector is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient mammal. Such as agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a change in the physiology of a recipient mammal. For example, in the treatment of retroviral infection, a compound which decreases the extent of

infection or of physiologic damage due to infection, would be considered therapeutically effective.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a

transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences. Various promoters may be used to drive vectors.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The present invention is directed to a novel method for enhancing locally the tumoricidal (or anti-angiogenic) properties of PDT (or heat) by using these therapeutic modalities to act as a molecular switch for the spatial and temporal expression of genes to enhance or act synergistically with the direct effects of PDT. The present invention is superior to current strategies which attempt to deliver localized cytotoxic gene therapy using constitutively-acting promoters, i.e. viral delivery systems using tissue-specific receptors or tissue-specific enhancers that limit gene transcription to a select group of cells. The temporal regulation of gene expression is not possible using constitutively-acting promoters due to the high basal expression of cytotoxic gene productions in normal cells and tissue. The present invention (combining inducible gene therapy and PDT or heat) enhances local tumor control without increasing normal tissue toxicity (increasing the therapeutic gain). Transcriptional

activation of a PDT or heat inducible promoter is controlled within a given tissue volume and for a specific time period. This procedure, therefore, exploits both the direct cytotoxicity induced by PDT or heat and the targeting potential of PDT or heat to induce spatial and temporal regulation of cytotoxic gene transcription.

PDT- or heat-targeted gene therapy uses a construct containing a PDT- or heat-inducible promoter upstream of a cDNA encoding a heterologous gene which is activated transcriptionally within the PDT or heat treatment field to enhance local cytotoxicity.

It is contemplated the present invention provides a method of temporal and localized treatment of a target tissue in an individual comprising the steps of: administering an expression vector to said individual, wherein said vector expresses a therapeutic, heterologous gene under control of a promoter inducible by photodynamic therapy or heat, and exposing said target tissue to said photodynamic therapy or heat.

For gene therapy applications, a person having ordinary skill in the art of molecular biology and oncology would be able to determine, without undue experimentation, the appropriate dosages and routes of administration to be employed in the novel method of the present invention.

The present invention describes a method for the selection and temporal expression of heterologous genes. The invention consists of two methods to obtain selective and temporal expression of heterologous genes both in cells grown in culture and for cells grown in-vivo as solid tumors. The human heat shock protein (hsp) promoter is used to express genes of interest under conditions of laser induced heating or Photodynamic Therapy- (PDT-) induced oxidative stress. Selective and temporal expression of heterologous genes (such as cytokines, toxins, tumor suppressor genes, antisense molecules and anti-angiogenic factors) are of significant therapeutic benefit in the treatment of tumors, vascular proliferation and tissue hypertrophy. Gene therapy targeted by laser induced heating, other heating sources (such as microwave, ultrasound or radiofrequency induced currents)

enhances treatment effectiveness by inducing expression of therapeutic genes in a controlled and localized manner.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion:

EXAMPLE 1

Cell Lines:

Radiation-induced fibrosarcoma cells (RIF) were used as parental control cells and as recipient cells transfected or transduced with hsp promoter reporter gene constructs. Three hsp promoter reporter gene cell lines were established and tested. Two of the cell lines were transfected with expression plasmids and one cell line was transduced with an inducible expression retroviral vector. Plasmids from StressGen Biotech Corp, p2500-CAT (providing inducible expression of chloramphenicol acetyl transferase (CAT) under the control of the hsp70 promoter) and p173OR (providing inducible expression of beta galactosidase (β-gal) under the control of the hsp70 promoter) as well as plasmid pMC1Neo from Stratagene (providing constitutive expression of the neomycin resistance gene under the control of a thymidine kinase promoter) were grown in supercompetent *E. coli*, isolated following alkaline lysis and purified. Each StressGen expression plasmid was co-transfected into RIF-1 cells along with pMC1Neo (5 to 1 ratio) using the calcium phosphate DNA precipitation technique. The cells were grown in 600 μg/ml G418 and resulting colonies were picked using cloning rings. G418 resistant clones were expanded and examined for β-gal or CAT activity using heat as a positive inducer. Positive clones were then tested for activity following PDT. Individual RIF-1 clones which expressed either β-gal or CAT activity following PDT were obtained.

The 3 cell lines used to prove that heat or PDT could be used as a switch for turning on heterologous reporter genes are:

1. **RIF HC-2 cells** which express CAT under the control of a 2.5 kb hsp promoter;
2. **RIF HB-3 cells** which express beta galactosidase under the control of a 2.5 kb hsp promoter; and

3. **RIF RHC-7 cells** which were obtained by transducing a modified GINA retroviral vector (containing a 300 bp hsp promoter ligated to the chloramphenicol acetyl transferase gene plus the phosphotransferase (neo resistance) gene constitutively expressed from the 5' LTR). Stable transfectants were established by growth in G-418 and then screened for heat induced expression of chloramphenicol acetyl transferase activity.

All three of these cell lines have stably integrated expression vectors and all cell lines are injected into the flank of C3H/HeJ mice to produce solid fibrosarcoma tumors.

EXAMPLE 2

TREATMENT CONDITIONS:

Hyperthermia treatment of cells in culture:

Cells ($2x10^6$) were seeded in T-75 flasks 24 hours prior to heat treatment. Hyperthermia was delivered by exposing cells to 45 °C in a temperature-controlled water bath.

Photosensitizer treatment of cells in culture (PDT):

Three different photosensitizers were examined: Photofrin (PH), mono-l-aspartyl chlorin e6 (Npe6), and tin etio purpurin (Sn ET2). Cells ($2x10^6$) were seeded in 100 mm Petri dishes 24 hours prior to a 16 hour incubation with one of the photosensitizers. Photosensitizer incubation concentrations were 25 μg/ml for PH and Npe6 and 0.75 μg/ml for SnET2. Cells were incubated with the photosensitizer in the dark for 16 hours in media containing 5% serum. The cells were then rinsed for 30 minutes in media containing 15% serum and then exposed to graded doses of red light. 664 nm diode laser light at a dose rate of 2 mW/cm^2 was used for Npe6 and Sn ET2. Broad-spectrum (570-650 nm) red light generated by a parallel series of 30-W fluorescent bulbs at a dose rate of 0.35 mW/cm^2 was used for cells incubated with PH.

Heat Treatment of tumors growing in mice:

Hyperthermia treatments of tumors measuring 6-7 mm in diameter consisted of a 20 minute exposure to 44.5-45 °C.

These temperatures were achieved by irradiating the tumors with a diode laser emitting 810 nm laser light at power density of 270 mW/cm^2.

PDT Treatment of tumors growing in mice:

Photodynamic therapy (PDT) treatments of tumors measuring 6-7 mm in diameter consisted of an intravenous injection of either PH or Npe6 at 5 mg/kg or SnET2 at 1.5 mg/kg. Non-thermal laser light exposure of the tumors was initiated either 4-5 hours (for Npe6) or 24 hours (for PH and Sn ET2) after drug administration. Red light at 630 nm was used for PH-mediated PDT and 664 nm light was used for Sn ET2- and Npe6-mediated PDT. Light dose rates were 75 mW/cm^2.

EXAMPLE 3

Cell and Tissue Beta-galactosidase assays:

Cell lysates (obtained by incubating the RIF HB-3 cells with a Reporter Lysis Buffer (Promega)) or tumor lysates (obtained by homogenizing RIF HB-3 solid tumors in Reporter Lysis Buffer with the Polytron) were placed in microcentrifuge tubes and centrifuged at 4°C for two minutes. 150 µl of the supernatant was tested for β-galactosidase activity by adding 150 µl of Assay 2X Buffer (Promega) containing the substrate ONPG (o-nitrophenyl-B-D-galactopyranoside) and incubating the reaction at 37°C for three hours. The reaction was stopped by adding 500 µl of 1 M Sodium Carbonate, and the absorbance at 420 nm was read with a spectrophotometer. Beta-galactosidase activity was expressed in milliunits/mg of protein (determined using the BIO-RAD protein assay).

EXAMPLE 4

Cell and Tissue CAT assays:

For cells, 10 µg of cellular protein (obtained by freeze/thawing of cells) was combined with 35 µl of 1M TrisCl pH 7.8, 10 µl of 6.0 mg/ml acetyl CoA, 2.5 µl of C-14 chloramphenicol (ICN Pharmaceuticals, Inc., Costa Mesa, CA, catalogue no. 12060)

and water to a final volume of 75 µl. The reaction mixture was incubated for 30 minutes in a 37° C water bath. Acetylated chloramphenicol was extracted from the reaction mixture by the addition of 1 ml ethyl acetate and vortexed for 1 minute and centrifuged at maximum speed for 5 minutes. The ethyl acetate layer was removed and dried in a speed vac for 45 minutes. Samples were dissolved in 30 µl of ethyl acetate, spotted on silica gel thin layer chromatography plate. TLC plates were developed in a chamber containing 200 ml of chloroform:methanol (95:5) for 45 minutes, air dried, and exposed to x-ray film overnight. Percent CAT conversion was calculated by cutting out the acetylated and non acetylated spots on the TLC plate and counted in a scintillation counter.

For tissue samples, CAT assays were performed according to "Molecular Cloning", Sambrook, Fritsch, Maniatis, Cold Spring Harbor Laboratory Press (1989), with slight modifications. 65.5 µg of protein sample was incubated at 65° C for 10 minutes to inactivate deacetylases. To each sample, 50 µl of 1 M TrisCl pH 7.8, 10 µl of 6.0 mg/ml acetyl CoA, 4.3 µl of C-14 chloramphenicol (ICN Pharmaceuticals, Inc., Costa Mesa, CA) and water were added to a final volume of 130 µl. Samples were incubated for 6 hours at 37° C and 5 µl of freshly made 6.0 mg/ml acetyl CoA solution was added to the samples every 2 hours. Following 6 hours of incubation, 1 ml of ethyl acetate was added to each sample, vortexed for 1 minute, centrifuged for 5 minutes, the organic layer was removed, air-dried in a speed vac, 30 µl of ethyl acetate was added to each sample, and the samples were spotted on to TLC plates. TLC plates were developed in a chamber containing 200 ml of chloroform:methanol (95:5) for 45 minutes, air dried and exposed to x-ray film overnight. Percent CAT conversion was calculated by cutting out the acetylated and non acetylated spots on the TLC plate and counted in a scintillation counter.

EXAMPLE 5

PDT inducible promoter/ TNF-α expression vector:

Starting plasmids are obtained from StressGen (p2500CAT) containing the hsp promoter and ATCC (pUC-R10173)

containing the complete coding sequence of TNF-α. The CAT gene from p2500CAT is removed by digestion with HindIII and BamH1. Resulting ends are modified using commercial adapters from New England Biolabs. The HindIII end is converted to EcoR1 using previously annealed adapters 1105 (EcoR1 - XmnI) and 1107 (HindIII - XmnI). The BamH1 end is converted to EcoR1 using adapters 1105 (EcoR1 - XmnI) and 1106 (BamH1 - XmnI). Following adapter insertion, the vector is ligated to the purified EcoR1 fragment of pUC-R10173. The circularized plasmid is used to transform competent *E.coli*. Minipreps of selected colonies are cut with AvaI and BglII to determine insert orientation. A colony with the correct insert is expanded and the resulting plasmid (pHspTNF) is transfected along with pMC1Neo in RIF cancer cells. Tumor cell lines containing the stably integrated hsp promoter/TNF-α expression vector, pHspTNF, is isolated.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method of enhancing the expression of a gene in a target tissue in an individual comprising the steps of:

delivering an expression vector to said target tissue in said individual, wherein said vector expresses a therapeutic, heterologous gene under control of a promoter inducible by photodynamic therapy or heat, wherein said gene is heterologous with regard to said promoter; and

exposing said target tissue to said photodynamic therapy or heat, wherein said photodynamic therapy or heat enhances the expression of said gene.

2. The method of claim 1, wherein said vector is administered systemically.

3. The method of claim 1, wherein said vector is administered locally.

4. The method of claim 1, wherein said vector is a retroviral vector, an adeno-associated viral vector, or a liposomal DNA vector.

5. The method of claim 1, wherein said promoter is a heat shock protein (hsp) promoter or a glucose regulated protein promoter.

6. The method of claim 1, wherein said heterologous gene is an immunomodulatory gene, a tumor suppressor gene, an anti-sense DNA or an anti-angiogenic gene.

7. The method of claim 6, wherein said immunomodulatory gene is a cytokine gene.

8. The method of claim 1, wherein said promoter is inducible by reactive oxygen species.

9. The method of claim 1, wherein said target tissue is a tumor, an area of abnormal tissue growth, or an area of abnormal blood vessel growth.

10. The method of claim 1, wherein said heat is generated by thermal laser, microwaves, ultrasound or radiofrequency waves.

11. The method of claim 1, wherein said heat generated is from 44.5°C to 45°C.

12. A method of enhancing the expression of a gene in a target tissue in an individual comprising the steps of:

administering a photosensitizer for photodynamic therapy to said individual;

delivering an expression vector to said target tissue in said individual, wherein said vector expresses a therapeutic, heterologous gene under control of a promoter inducible by photodynamic therapy, wherein said gene is heterologous with regard to said promoter;

allowing said photosensitizer and said expression vector to be taken up by said target tissue; and

exposing said target tissue to light, wherein said light combined with said photosensitizer will generate reactive oxygen species to induce said promoter inducible by photodynamic therapy, enhancing said therapeutic, heterologous gene expression.

13. The method of claim 12, wherein said photosensitizer is selected from the group of photofrin, tin

etiopurpurin, mono-l-aspartyl chlorin e6, benzoporphyrin derivative, meso-tetra-(hydroxyphenyl)chlorin and 5-amino levulinic acid.

14. The method of claim 12, wherein said promoter is a heat shock protein (hsp) promoter or a glucose regulated protein promoter.

15. The method of claim 12, wherein said vector is a retroviral vector, an adeno-associated viral vector, or a liposomal DNA vector.

16. The method of claim 12, wherein said heterologous gene is an immunomodulatory gene, a tumor suppressor gene, an anti-sense DNA or an anti-angiogenic gene.

17. The method of claim 16, wherein said immunomodulatory gene is a cytokine gene.

18. The method of claim 12, wherein said light is generated by a laser.

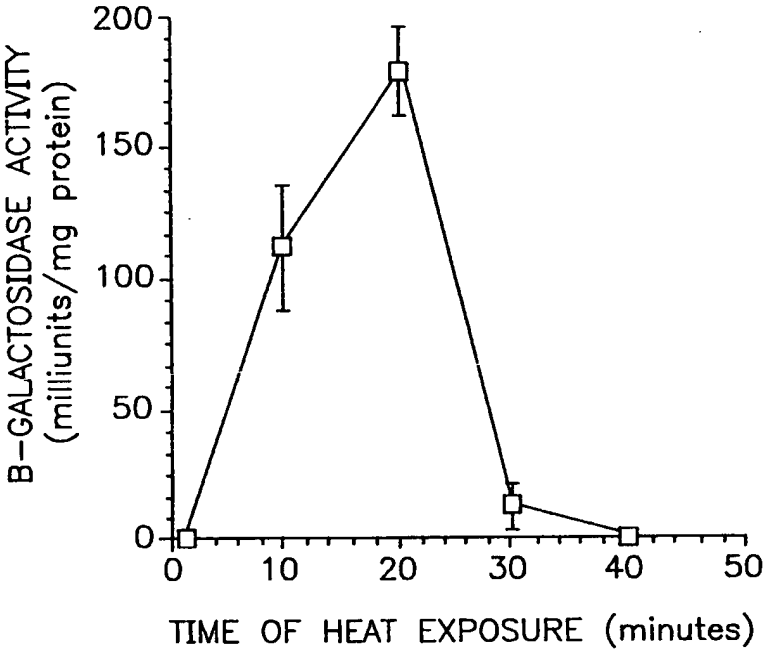
B-GALACTOSIDASE ACTIVITY
(milliunits/mg protein)
200
150
100
50
0
0
10
20
30
40
50
TIME OF HEAT EXPOSURE (minutes)

FIG. 1

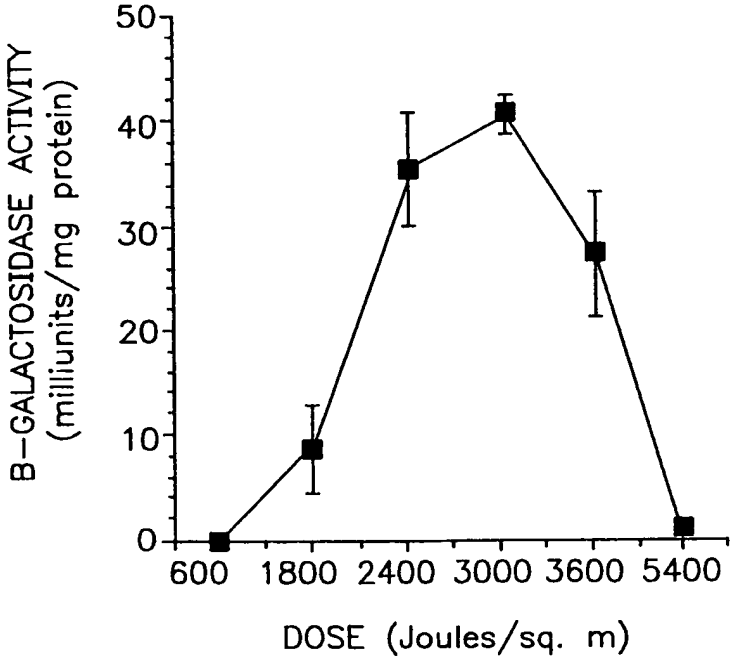
B-GALACTOSIDASE ACTIVITY
(milliunits/mg protein)
50
40
30
20
10
0
600
1800
2400
3000
3600
5400
DOSE (Joules/sq. m)

FIG. 2

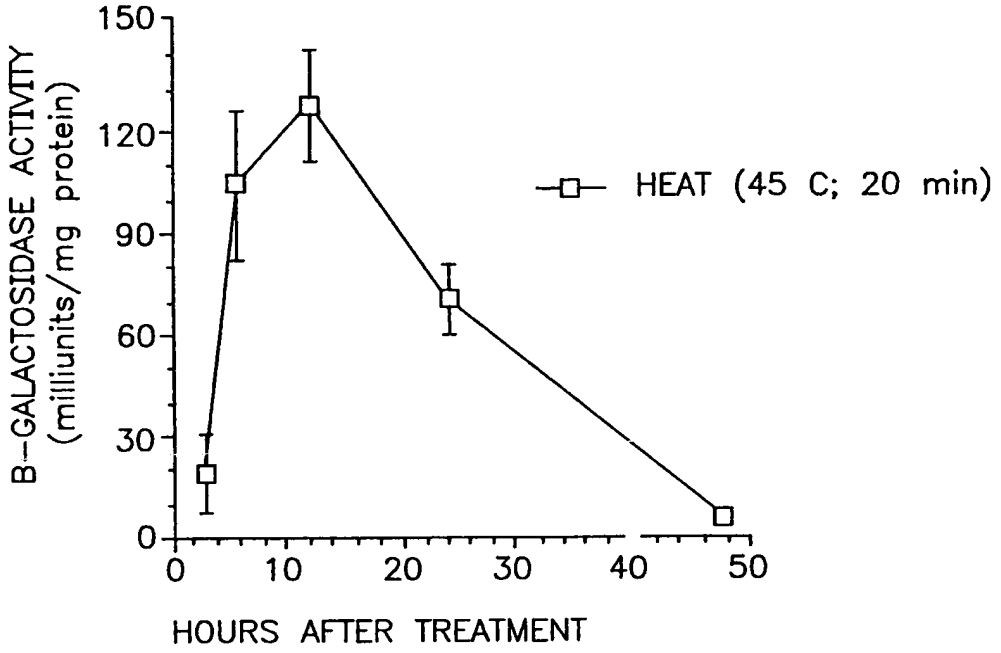
B-GALACTOSIDASE ACTIVITY
(milliunits/mg protein)
150
120
90
60
30
0
0
10
20
30
40
50
HOURS AFTER TREATMENT
HEAT (45 C; 20 min)

FIG. 3

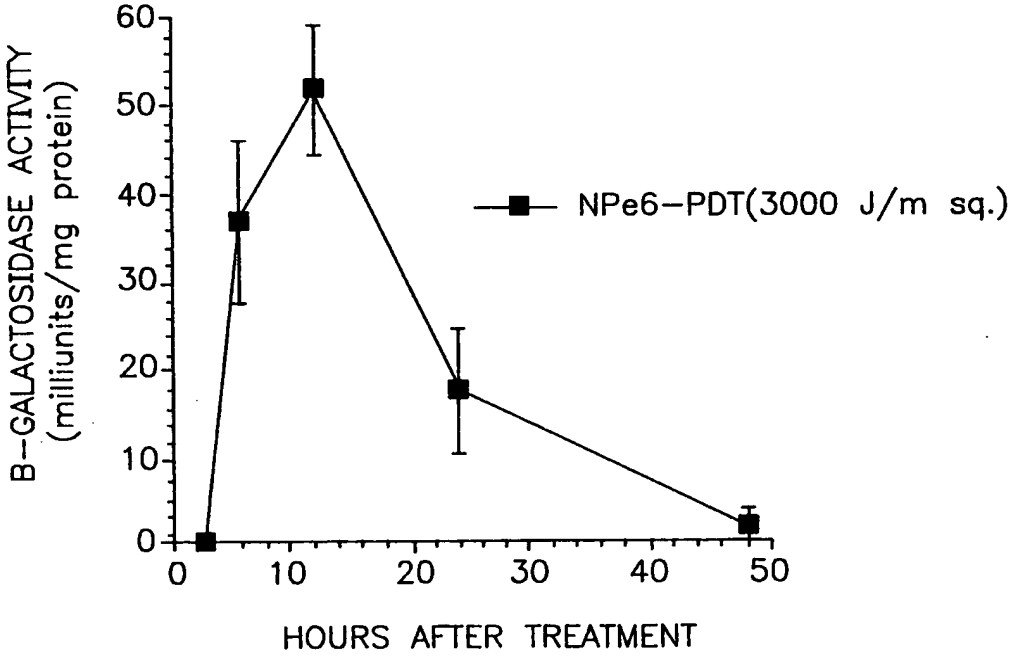
B-GALACTOSIDASE ACTIVITY
(milliunits/mg protein)
60
50
40
30
20
10
0
0
10
20
30
40
50
HOURS AFTER TREATMENT
NPe6-PDT(3000 J/m sq.)

FIG. 4

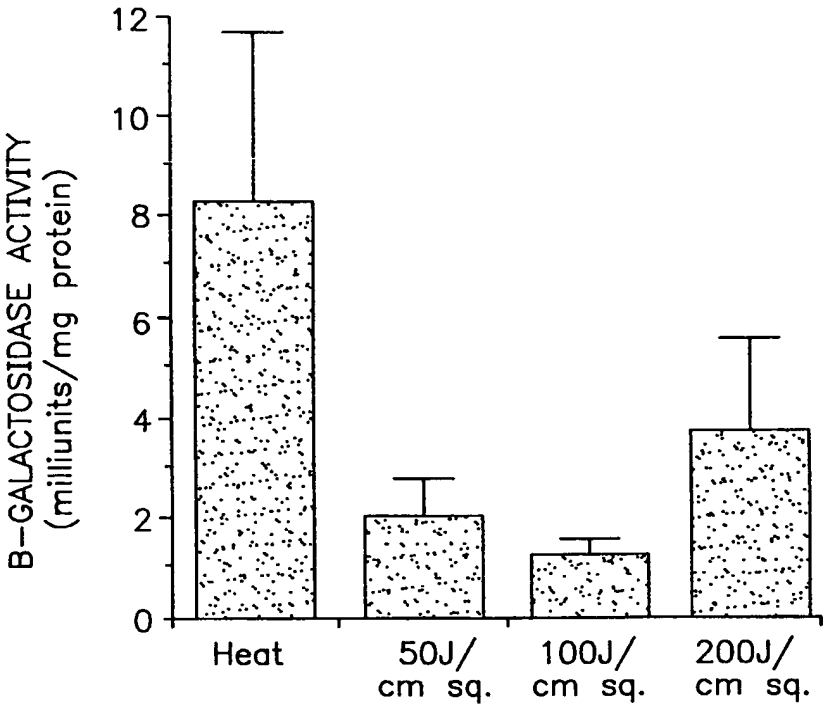
B-GALACTOSIDASE ACTIVITY
(milliunits/mg protein)
12
10
8
6
4
2
0
Heat
50J/
cm sq.
100J/
cm sq.
200J/
cm sq.

FIG. 5

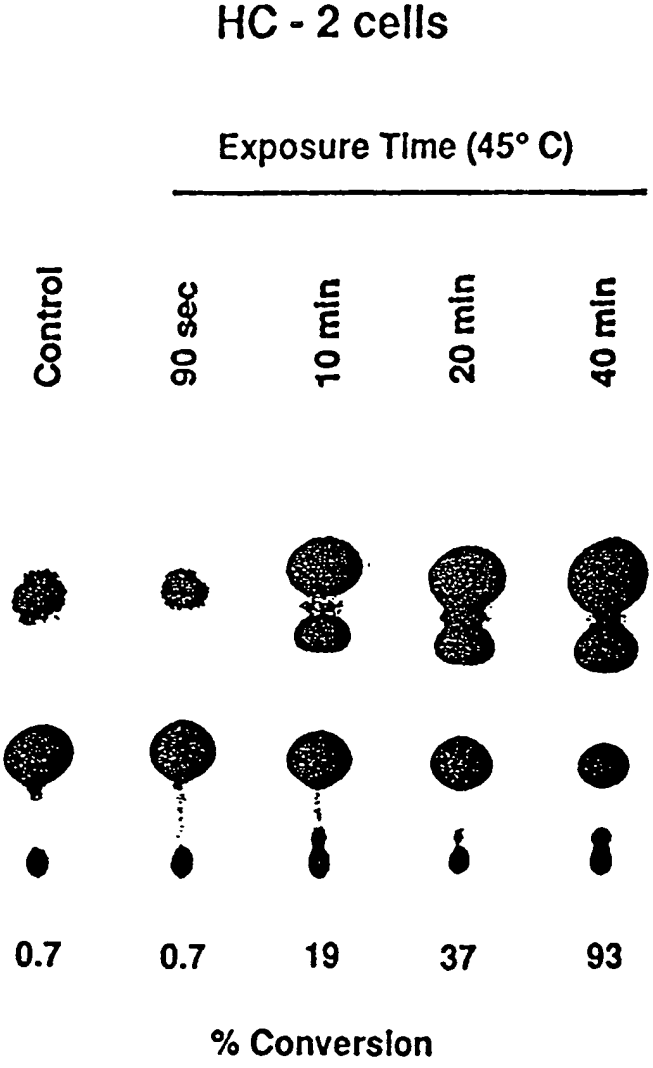
HC - 2 cells
Exposure Time (45° C)
Control
90 sec
10 min
20 min
40 min
0.7
0.7
19
37
93
% Conversion

FIGURE 6

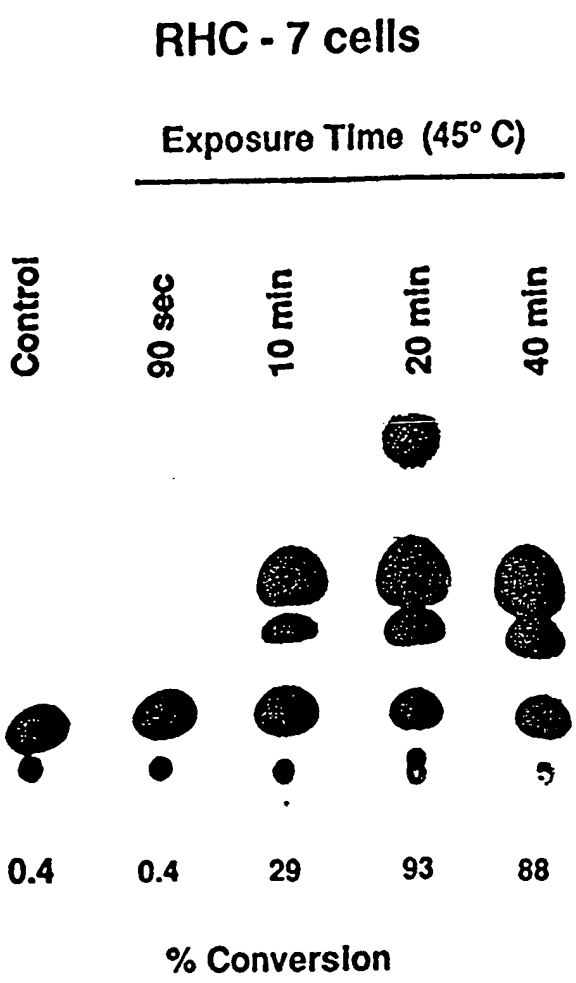
RHC - 7 cells
Exposure Time (45° C)
Control
90 sec
10 min
20 min
40 min
0.4
0.4
29
93
88
% Conversion

FIGURE 7

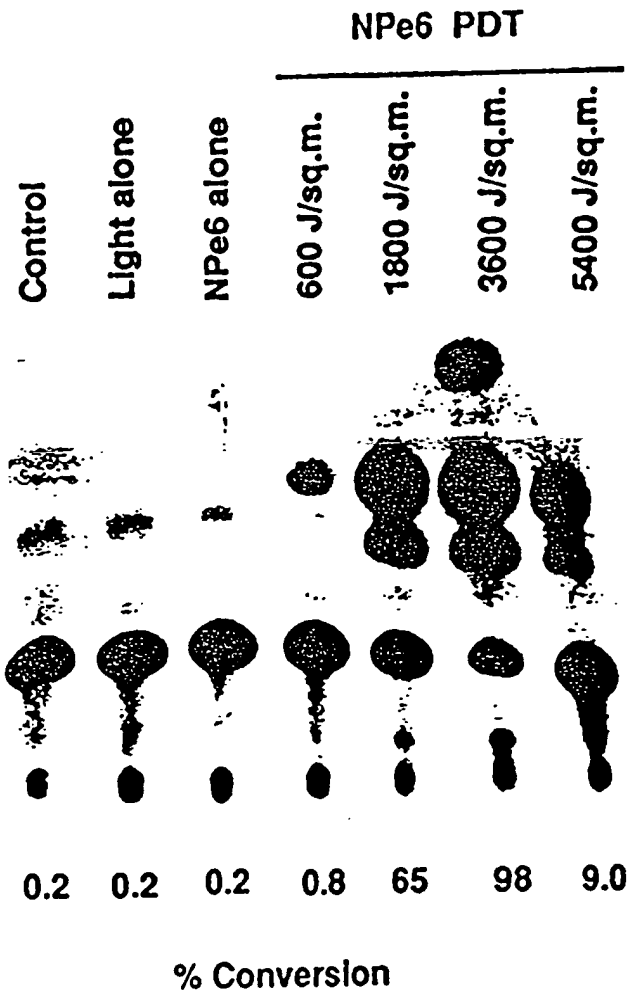
HC - 2 cells
NPe6 PDT
Control
Light alone
NPe6 alone
600 J/sq.m.
1800 J/sq.m.
3600 J/sq.m.
5400 J/sq.m.
0.2
0.2
0.2
0.8
65
98
9.0
% Conversion

FIGURE 8

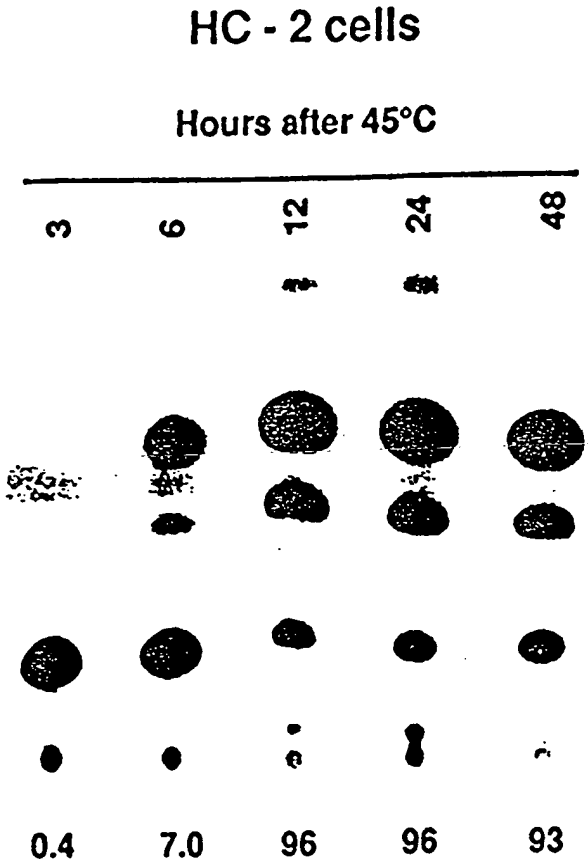

FIGURE 9

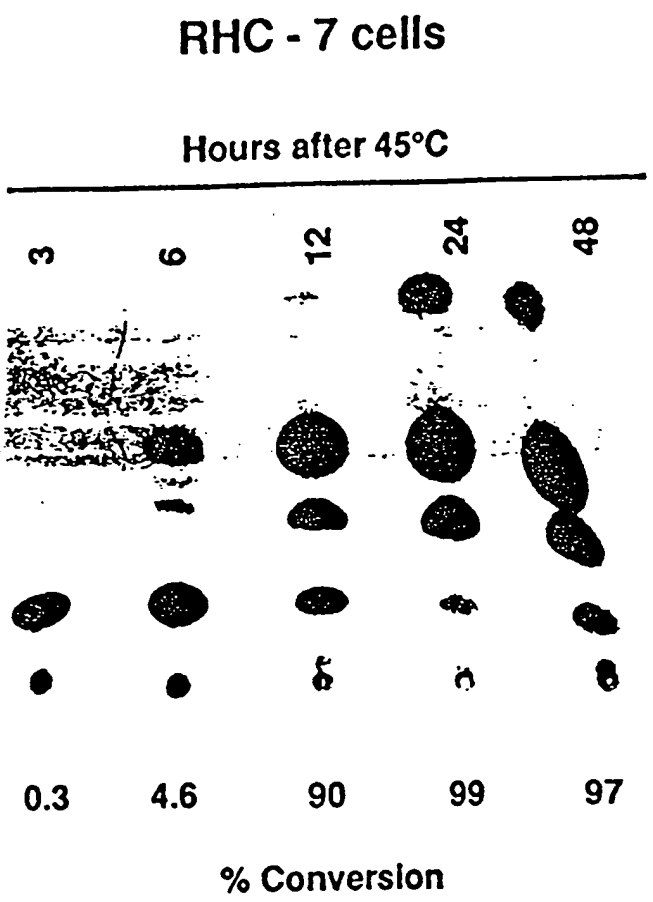
RHC - 7 cells
Hours after 45°C
3
6
12
24
48
0.3
4.6
90
99
97
% Conversion

FIGURE 10

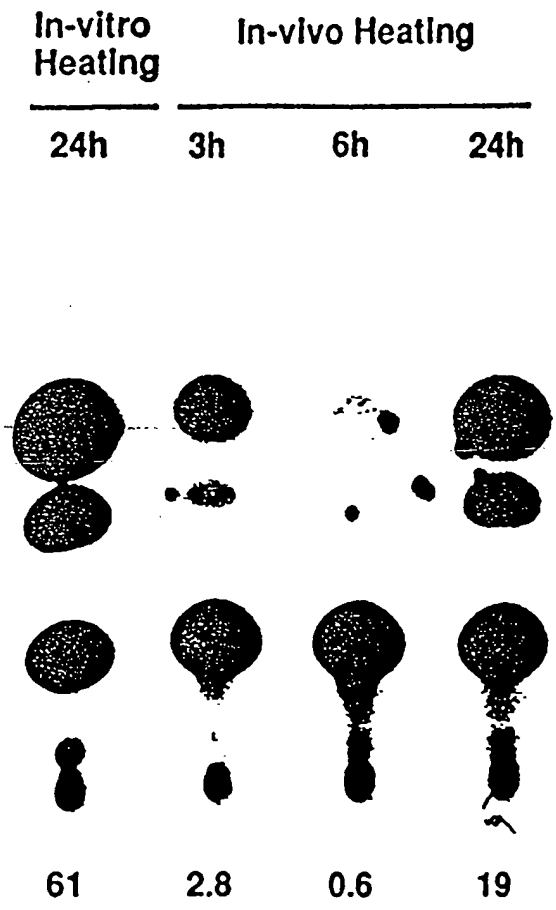
In-vitro Heating
In-vivo Heating
24h
3h
6h
24h
61
2.8
0.6
19
% Conversion

FIGURE 11

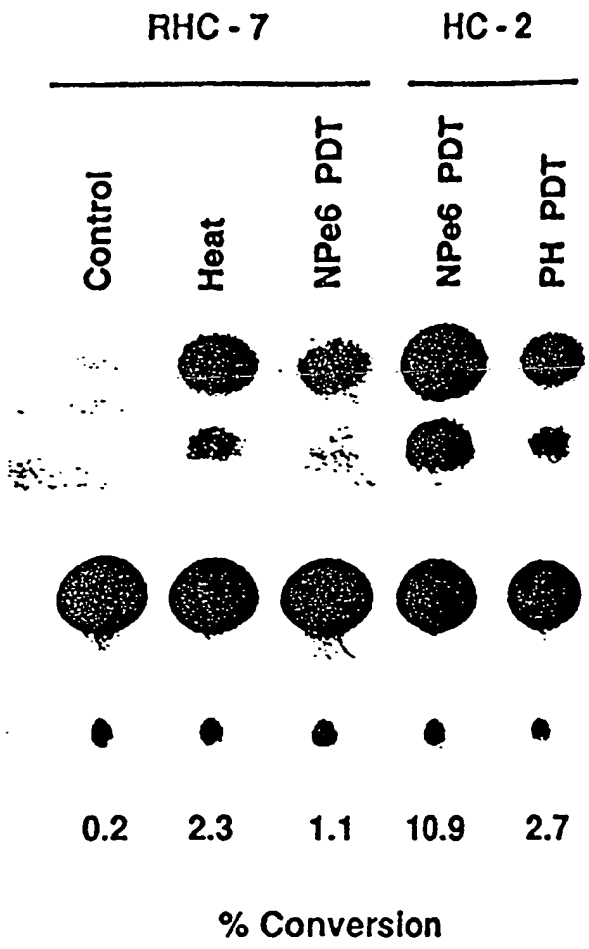
RHC - 7
HC - 2
Control
Heat
NPe6 PDT
NPe6 PDT
PH PDT
0.2
2.3
1.1
10.9
2.7
% Conversion

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04551

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; C12N 13/00, 15/00
US CL :514/44; 435/172.1, 172.3, 173.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/172.1, 172.3, 173.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT BIOTECHNOLOGY, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,571,797 A (OHNO et al) 05 November 1996, see the entire document.	1-18
P, Y	US 5,612,318 A (WEICHSELBAUM et al) 18 March 1997, see the entire document.	1-18
Y	LEWANDOWSKI et al. Alteration of Gene Expression by Hyperthermia Potentiated Photodynamic Therapy With Aluminum Phthalocyanine Tetrasulfonate on Normal and Malignant Human Cells In Vitro. Clinical Research. 1991, Vol. 39, No. 2, page 547A, see the entire abstract.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 APRIL 1998	20 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRUCE CAMPELL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04551

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CURRY et al. Stress Protein Expression in Murine Tumor Cells Following Photodynamic Therapy with Benzoporphyrin Derivative. Photochemistry and Photobiology. 1993, Vol. 58, No. 3, pages 374-379, see the entire document.	1-18
Y	GOMER et al. Glucose Regulated Protein Induction and Cellular Resistance to Oxidative Stress Mediated by Porphyrin Photosensitization. Cancer Research. 15 December 1991, Vol. 51, pages 6574-6579, see the entire document.	1-18
Y	LUNA et al. Photodynamic Therapy Mediated Induction of Early Response Genes. Cancer Research. 01 March 1994, Vol. 54, pages 1374-1380, see the entire document.	1-18
Y	O'ROURKE et al. X-irradiation- and carcinogen-induced proteins in cultured CHO cells. Biochemical Society Transactions. 1991, Vol. 20, page 74S, see the entire document.	1-18